C-Reactive Protein Inhibits Endothelium-Dependent Nitric Oxide-Mediated Dilation of Retinal Arterioles via Enhanced Superoxide Production

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PURPOSE. Elevated levels of C-reactive protein (CRP), a proinflammatory marker, are associated with systemic vascular disorders. In addition, clinical studies have implicated that elevated CRP is an independent risk factor for diabetic retinopathy and age-related macular degeneration. However, the direct effect of CRP on ocular microvascular reactivity remains unknown. The authors examined whether CRP can affect endothelium-dependent nitric oxide (NO)-mediated dilation of retinal arterioles and whether oxidative stress and distinct protein kinase signaling pathways are involved in the CRP-mediated effect.

METHODS. Porcine retinal arterioles (internal diameter, 71 ± 2 μm) were isolated and pressurized without flow for in vitro study. Diameter changes were recorded using videomicroscopic techniques. Dihydroethidium (DHE) was used to detect superoxide production.

RESULTS. Intraluminal treatment with a clinically relevant concentration of CRP (7 μg/mL, 60 minutes) significantly attenuated arteriolar dilation to endothelium-dependent NO-mediated agonists bradykinin and A23187 but not to endothelium-independent NO donor sodium nitroprusside. In the presence of superoxide scavenger TEMPO1, NAD(P)H oxidase inhibitor apocynin, p38 kinase inhibitor SB203580, simvastatin, or Rho-kinase inhibitor Y-27632, the detrimental effect of CRP on bradykinin-induced dilation was prevented. DHE staining showed that CRP produced TEMPO-sensitive superoxide production in the arteriolar endothelium.

CONCLUSIONS. CRP inhibits endothelium-dependent NO-mediated dilation in retinal arterioles by producing superoxide from NAD(P)H oxidase, which appears to be linked with p38 kinase and RhoA/Rho-kinase activation. By impairing endothelium-dependent NO-mediated vasoreactivity, CRP can potentially facilitate the development of retinal vascular diseases. In addition, statins are beneficial by preserving endothelial function, possibly through inactivation of the RhoA/Rho-kinase pathway. (Invest Ophtalmol Vis Sci. 2008;49:2053–2060) DOI: 10.1167/iovs.07-1387

C-reactive protein (CRP), a biochemical marker of inflammation, has recently been recognized as a strong predictor of cardiovascular risk1,2 and a possible direct mediator of the disease process because of its numerous proatherogenic effects on vascular cells.3 These properties include reduction of endothelial nitric oxide synthase (eNOS) activity4,5 and nitric oxide (NO) release from cultured endothelial cells,6 upregulation of endothelial adhesion molecules7,8 and generation of reactive oxygen species from endothelial9 and smooth muscle10 cells. Recent clinical studies have implicated a pathogenic role of CRP in various cardiovascular disorders (for a review, see Verma et al.11) and its possible association with ocular diseases such as polypoidal choroidal vasculopathy,12 age-related macular degeneration (AMD),13–15 and diabetic retinopathy.16 Although endothelial dysfunction16,17 and inflammation17–21 have been reported to contribute to retinal vascular abnormalities of diabetic retinopathy, the possible direct impact of CRP on vasomotor function of the retinal microvasculature has not been documented. We have previously shown that systemic administration of simvastatin can increase blood flow in retinal arteries and veins in healthy humans,22 possibly through endothelium-dependent, NO-mediated dilation of small retinal arterioles.23 In addition, statins protect vascular function from oxidative insult.24 These studies suggest that statins may have therapeutic potential for improving endothelium-dependent vasomotor function in retinal microvasculature. Therefore, in the present study, we tested the hypothesis that elevation of the intravascular level of CRP can lead to endothelial dysfunction and impair vasodilation by reducing NO bioavailability through enhanced oxidative stress. We also hypothesize that the administration of simvastatin can preserve endothelium-dependent NO-mediated vasodilation in retinal arterioles. In addition, the roles of inflammatory signaling cascades such as p38 kinase and RhoA/Rho-kinase in the detrimental effect of CRP were examined. To eliminate the confounding effects (i.e., neurohumoral and hemodynamic factors) commonly encountered in in vivo preparations, we used an isolated vessel approach to directly assess the effect of CRP on endothelial and vasomotor function of retinal arterioles.

METHODS

Animal Preparation

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Scott & White Institutional Animal Care and Use Committee. Pigs (8–12 weeks old of either sex; 7–10 kg) purchased from Barfield Farms (Rogers, TX) were sedated with tiletamine/zolazepam (4.4 mg/kg, intramuscularly; Telazol; Wyeth, Philadelphia, PA) and xylazine (2.2 mg/kg, intramuscularly), anesthetized with so-
durom pentobarbital (30 mg/kg, intravenously), intubated, and ventilated with room air. Heparin (1000 U/kg) was administered into the marginal ear vein to prevent clotting, and the eyes were enucleated and immediately placed in a moist chamber on ice.

**Isolation and Cannulation of Microvessels**

The preparation of isolated retinal arterioles has been described in our previous studies. In brief, the anterior segment and vitreous body were removed carefully under a dissection microscope. The eyecup was placed in a cooled dissection chamber (approximately 8°C) containing physiologic salt solution (PSS; NaCl, 145.0 mM; KCl, 4.7 mM; CaCl₂, 2.0 mM; MgSO₄, 1.17 mM; NaH₂PO₄, 1.2 mM; glucose, 5.0 mM; pyruvate, 2.0 mM; EDTA, 0.02 mM; and MOPS, 3.0 mM) with 1% albumin (USB, Cleveland, OH). Second-order retinal arterioles (40–60 μm in internal diameter in situ; 0.6–1.0 mm in length without branches) were carefully dissected out using a Vannas spring scissors and a pair of microdissection forceps (Dumont; Fine Science Tools, Foster City, CA) with the aid of a stereomicroscope (model SZX12; Olympus, Melville, NY). After careful removal of any remaining neural/vascular tissues, the arteriole was transferred for cannulation to a polymethylmethacrylate vessel chamber containing PSS-albumin solution equilibrated with room air at ambient temperature. Both ends of the arteriole were cannulated with glass micropipettes (tip outer diameter, 50–40 μm) filled with PSS-albumin solution, and the outside of the arteriole was securely tied to the pipettes with 11–0 ophthalmic suture (Alcon, Fort Worth, TX). After cannulation, the vessel and pipettes were transferred to the stage of an inverted microscope (model CKX41; Olympus) coupled to a video camera (Sony DXC-190; Labtek, Campbell, CA) and video micrometer (Cardiovascular Research Institute; Texas A&M Health Science Center, College Station, TX) for continuous measurement of the internal diameter. The micropipettes were connected to independent pressure reservoirs (i.e., 30-mL glass syringes with 10 mL PSS). By adjusting the height of the reservoirs, the vessel was pressurized to 55 cm H₂O (approximately 40 mm Hg) intraluminal pressure without flow based on pressure ranges documented in retinal arterioles in vivo and in the isolated, perfused retinal microcirculation. Preparations with leaks were excluded from further study.

**Experimental Protocols**

The human recombinant CRP (Calbiochem, San Diego, CA) used in the following protocols was initially dialyzed for 24 hours against Dulbecco PBS using a dialysis slide (Pierce, Rockford, IL) with a cutoff of 10 kDa to remove sodium azide, which is present as a preservative in commercial preparations of CRP. Endotoxin, which can affect endothelial function, was also removed from the CRP by using detoxigel columns (Pierce) and was found to be less than 0.06 EU/mL by the Limulus assay (Cambrex, East Rutherford, NJ).

Cannulated arterioles were bathed in PSS at 36°C to 37°C to allow the development of basal tone. After vessels developed a stable basal tone (approximately 30–40 minutes), we assessed the effect of CRP on NO-mediated vasodilation. For this purpose, the endothelium-dependent NO-mediated vasodilation to bradykinin (1 × 10⁻¹² to 1 × 10⁻⁸ M) was established before and after 30-minute intraluminal incubation with either a subclinical (0.7 μM) or 60-minute intraluminal incubation with a clinically relevant (7 μg/mL) concentration of CRP. The vehicles were exposed to each concentration of bradykinin for 2 to 3 minutes until a stable diameter was established. In our preliminary study, bradykinin-induced dilation was reproducible and did not deteriorate after repeated application (n = 8).

To determine whether the effect of CRP is selective toward endothelium-dependent NO-mediated dilation, dose-dependent responses to receptor-independent but endothelial NO-mediated vasodilator A23187 (1 × 10⁻⁸ to 5 × 10⁻⁶ M) or to endothelium-independent NO donor sodium nitroprusside (1 × 10⁻⁸ to 1 × 10⁻⁴ M) were established before and after 60-minute intraluminal incubation with 7 μg/mL CRP in another series of experiments. In some vessels, the role of NO in the A23187-induced dilation was verified after 30-minute extraluminal incubation with LNAME (10 μM).

The roles of superoxide and oxidative enzymes NAD(P)H oxidase and xanthine oxidase in mediating the effect of CRP were determined in a separate group of vessels by examining bradykinin-induced dilation combined with cell-permeable superoxide scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; 1 mM), NADPH oxidase inhibitor apocynin (100 μM), or xanthine oxidase inhibitor allopurinol (10 μM). The role of stress-activated protein kinases was examined by treating the vessels with CRP combined with p38 kinase inhibitor SB203580 (0.1 μM; Calbiochem).

To assess the ability of simvastatin to mitigate the effect of CRP on NO-mediated vasodilation, vessels were treated with CRP (7 μg/mL) combined with a clinical dose (10 mM) of simvastatin (intraluminal; Merck Research Laboratories, West Point, PA). In addition, the role of the RhoA/Rho-kinase pathway in mediating the CRP effect was examined by treating another group of vessels with CRP in combination with Rho-kinase inhibitor Y-27632 (0.1 μM).

Drugs were obtained from Sigma-Aldrich (St. Louis, MO) and were dissolved in PSS except when specifically stated otherwise. Simvastatin was generously provided by Merck Research Laboratories and activated by alkaline hydrolysis according to the manufacturer’s instructions. Simvastatin was dissolved in ethanol, and the final concentration of ethanol in the vessel bath was less than 0.1%. Vehicle control studies indicated that this concentration of ethanol had no effect on arteriolar function.

**Detection of Superoxide**

Superoxide production in isolated retinal arterioles was evaluated with the fluorescent dye dihydroethidium (DHE). Isolated and pressurized retinal arterioles (70–100 μm in diameter and 1.5 mm in length) were incubated intraluminally with PSS containing vehicle, CRP (7 μg/mL), or CRP plus TEMPOL (1 mM) at 37°C for 60 minutes and then were stained with DHE (4 μM) for 30 minutes. After they were washed, arterioles were embedded in OCT compound (Tissue-Tek; Electron Microscopy Sciences, Hatfield, PA) for cryostat sections. Embedded arterioles were cut into 12-μm-thick sections and placed on glass slides. Images were taken with a fluorescence microscope (Axiovert 200c; Zeiss). Fluorescence was detected with a 620/60 (590–650 nm) bandpass emission filter. Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues.

**Statistical Analysis**

At the end of each experiment, the vessel was relaxed in an EDTA (1 mM)-calcium-free PSS to obtain its maximal diameter at 55 cm H₂O intraluminal pressure. All diameter changes in response to agonists were normalized to this maximal vasodilation and expressed as a percentage of maximal dilation. Data are reported as mean ± SEM, and n values represent the number of vessels studied. Statistical comparisons of vasodilator responses were performed by two-way analysis of variance followed by the Bonferroni multiple-range test. Changes in resting tone by CRP or pharmacologic inhibitors were analyzed by paired Student’s t-test. P < 0.05 was considered significant.

**Results**

**Effect of CRP on NO-Mediated Vasodilation**

In this study, all vessels (n = 49) developed a similar level of basal tone (constricted to 68% ± 2% of maximal diameter) at 36°C to 37°C bath temperature with 55 cm H₂O intraluminal pressure. Average resting and maximal diameters of the vessels were 71 ± 2 μm and 105 ± 2 μm, respectively. Bradykinin

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**References**


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**Figure Captions**

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**Table 1**

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**Author Contributions**

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did not cause further reduction of bradykinin-induced dilation (data not shown). CRP at 7 µg/mL also significantly reduced A23187-induced vasodilation (Fig. 2B). On the other hand, the dilation of retinal arterioles to the endothelium-independent NO donor sodium nitroprusside was not affected by CRP (7 µg/mL) (Fig. 2C).

Roles of Superoxide, NAD(P)H Oxidase, Xanthine Oxidase, and p38 Kinase

To determine whether superoxide production is involved in the impairment of bradykinin-induced vasodilation, vessels were treated with CRP in the presence of a membrane-permeable superoxide scavenger TEMPOL or of specific oxidase inhibitors. In the presence of TEMPOL, the inhibition of bradykinin-induced vasodilation by CRP (7 µg/mL) was prevented (Fig. 3A). This preventive effect was also found in vessels treated with NAD(P)H oxidase inhibitor apocynin (Fig. 3B). However, xanthine oxidase inhibitor allopurinol had no influence on the CRP-mediated effect (Fig. 3B). The basal tone was not significantly altered by TEMPOL (control, 64% ± 4%; TEMPOL, 64% ± 4%; P = 0.94), apocynin (control, 65% ± 6% vs. apocynin, 63% ± 7%; P = 0.74) or allopurinol (control, 60% ± 2% vs. allopurinol, 57% ± 5%; P = 0.67). In the presence of p38 kinase inhibitor SB203580, the inhibitory effect of CRP on vasodilation to bradykinin was also prevented (Fig. 4). The basal tone was not significantly altered by SB203580 (control, 63% ± 5% vs. SB203580, 62% ± 6%; P = 0.84). In another group of vessels, dilation to bradykinin (1 nM) was not altered after 60-minute intraluminal administration of TEMPOL (control, 54% ± 12% dilation vs. TEMPOL, 56% ± 14%; n = 3; P = 0.65) or SB203580 (control, 55% ± 11% dilation vs. SB203580, 51% ± 6%; n = 3; P = 0.61) alone.

Effect of Simvastatin and RhoA/Rho-Kinase Blockade

Coadministration of CRP and simvastatin prevented the detrimental action of CRP on bradykinin-induced vasodilation (Fig. 5A). Similarly, the inhibitory effect of CRP on the vasodilatory response to bradykinin was abolished in the presence of Rho-kinase inhibitor Y-27632 (Fig. 5B). The basal tone was not significantly altered by simvastatin (control, 59% ± 3% vs. simvastatin, 55% ± 3%; P = 0.24) or Y-27632 (control, 62% ± 2% vs. Y-27632, 59% ± 2%; P = 0.50). In addition, the vasodilatory response to bradykinin (1 nM) was not altered after 60-minute intraluminal treatment with simvastatin (control, 63% ± 10% dilation vs. simvastatin, 59% ± 12%; n = 5; P = 0.43) or Y-27632 (control, 60% ± 9% dilation vs. Y-27632, 57% ± 10%; n = 3; P = 0.23) alone.

Effect of CRP on Superoxide Production

In the absence of CRP (i.e., vehicle control), DHE fluorescence revealed sparse levels of superoxide in the vessel wall (Fig. 6). In contrast, intraluminal incubation of vessels with CRP (7 µg/mL; 60 minutes) markedly increased the superoxide level in the endothelial layer. Endothelial and smooth muscle layers were identified by setting the scanning threshold to obtain a clear background image of the vessel wall. TEMPOL markedly reduced the CRP-induced fluorescent signals for superoxide in the endothelium (Fig. 6).

DISCUSSION

The inflammatory marker CRP has recently been shown to be an independent risk for cardiovascular and peripheral arterial disease11 and a pathogenic factor leading to endothelial dysfunction in the cell culture model.38,39 Moreover, elevated
levels of CRP have been prospectively associated with an increased risk for hypertension and for type 1 and type 2 diabetes mellitus. Because hypertension and diabetes are major risk factors for vascular disease, we investigated whether CRP may play a role in retinal vascular dysfunction.

**Figure 2.** Effect of CRP on retinal vascular reactivity. (A) Dilation of isolated retinal arterioles to bradykinin was examined before and after intraluminal incubation with 7 μg/mL CRP (n = 7) or 0.7 μg/mL CRP (n = 5) for 60 minutes. Retinal arteriolar dilation to A23187 (B, n = 5) and sodium nitroprusside (C, n = 5) was examined before and after intraluminal incubation with 7 μg/mL CRP for 60 minutes. *P < 0.05 vs. Control.

**Figure 3.** Blockade of superoxide production or NAD(P)H oxidase activation prevents CRP-induced reduction of retinal arteriolar dilation to bradykinin. (A) Dilation of retinal arterioles to bradykinin was examined before (Control, n = 5) and after intraluminal incubation with 7 μg/mL CRP plus superoxide anion scavenger TEMPOL (1 mM; n = 5). (B) Dilation of retinal arterioles to bradykinin was examined before (Control, n = 10) and after intraluminal incubation with 7 μg/mL CRP plus NAD(P)H oxidase inhibitor apocynin (100 μM; n = 5) or xanthine oxidase inhibitor allopurinol (10 μM; n = 5). *P < 0.05 vs. Control.
risk factors for retinal vascular disorders and their associations with inflammation and endothelial dysfunction have been suggested in humans with retinopathy, it is important to evaluate the direct effect of CRP on retinal microvascular function. Unfortunately, there has been no study hitherto to document the direct effect of CRP on retinal vasomotor function. The present study is the first to show that CRP significantly reduces the dilations of retinal arterioles to bradykinin and A23187 but not to sodium nitroprusside, suggesting that CRP compromises retinal endothelial function in terms of NO-mediated vasodilation.

It has been suggested that a serum CRP level lower than 1 μg/mL is considered a low cardiovascular risk for coronary artery disease. On the other hand, CRP levels between 1 and 3 μg/mL are interpreted as intermediate risks for cardiovascular events, and levels between 3 and 10 μg/mL indicate high risk. In the study by Verma et al., only CRP concentrations greater than 3 μg/mL significantly reduced NO release from cultured human umbilical vein endothelial cells. Several recent clinical studies suggest a close association between serum CRP and ocular vascular disorders related to AMD and diabetic retinopathy. It has been reported that patients with the highest quartile of CRP (greater than 6.5 μg/mL) exhibit a high risk for AMD. In addition, a more than threefold higher incidence of AMD was found in women with serum CRP levels greater than 5 μg/mL. In another clinical study, the prevalence of diabetic retinopathy was reported to be increased with higher tertiles of CRP (highest tertile range, 3–35 μg/mL). The concentrations of CRP used in the present study (0.7 and 7 μg/mL) covered the physiological and pathophysiological ranges, and only the high level of CRP exhibited inhibitory action on endothelium-dependent vasomotor function. It appears that CRP levels known to predict cardiovascular events produce adverse effects on endothelial function in the retinal microvasculature. This is consistent with the findings recently reported in coronary arterioles.

In addition to the reduction of bradykinin-induced NO-mediated dilation, CRP reduced the vasodilation of retinal arterioles in response to the calcium ionophore A23187, which is known to activate eNOS by the elevation of intracellular calcium independent of receptor activation. This contention is supported by the present finding that A23187-elicted dilation of retinal arterioles was abolished by the NOS inhibitor. It has been reported that CRP (more than 10 μg/mL) decreases enzyme activity of eNOS in cultured human aortic endothelial cells. Moreover, in addition to the previously known action of CRP to attenuate eNOS expression after prolonged exposure
dilatory function in the absence of CRP but did prevent the study, p38 kinase blockade did not influence the normal vaso-
coronary artery disease.48 Our findings suggest that the detri-
linked in series with Rho-kinase activation leading to NAD(P)H oxidase activation and endothelial production of superoxide. On the other hand, Rho-kinase blockade inhibits NAD(P)H oxidase activation.55 These results suggest that the RhoA/Rho-kinase pathway may be involved in the development of oxidative stress through the activation of NAD(P)H oxidase. In the present study, inhibition of Rho-kinase preserved NO-mediated dilation to bradykinin, suggesting the involvement of RhoA/Rho-kinase activation in CRP-induced endothelial dysfunction in retinal arterioles. Our results are consistent with recent evidence showing that CRP can activate RhoA/Rho-kinase signaling in cultured bovine aortic endothelial cells.56,57 Because both NAD(P)H oxidase and Rho-kinase blockade prevented the detrimental actions of CRP, it is possible that these events are linked in series with Rho-kinase activation leading to NAD(P)H oxidase activation. Alternatively, the inhibition of eNOS function might be involved because RhoA/Rho-kinase activation has been shown to negatively regulate eNOS activity and NO production in cultured human endothelial cells.58 It does not appear that Rho-kinase activation alters eNOS function in the absence of CRP because bradykinin-induced vasodilation remained normal in the presence of Y-27632 alone. Future studies are required to determine the precise series of events leading to NAD(P)H oxidase activation and RhoA/Rho-kinase signaling by CRP.

Reductions in inflammation and oxidative stress or inhibi-
tion of RhoA/Rho-kinase activity by statins have been reported to improve endothelial function.59,60 In addition, we have recently demonstrated that simvastatin can elicit the dilation of retinal arterioles through NO production from endothelium by inhibiting RhoA/Rho-kinase signaling.23 Therefore, it is reasonable to consider whether statins can prevent CRP-induced retinal vascular dysfunction. Interestingly, Tan et al.61 reported that atorvastatin treatment in patients with type 2 diabetes led to a reduction in plasma CRP and an improvement in endothelium-dependent vasodilation. However, it is unclear whether this beneficial effect is the result of a direct action of statin or the secondary effect of CRP lowering. In the present study, we found that a clinical dose (10 nM) of simvastatin prevented the inhibitory effect of CRP on endothelial function. This protective effect may be related to the reduction of oxidative stress because simvastatin has been shown to reduce superoxide formation in the rat coronary artery endothelial cells in cul-
tured. In addition, aortic and renal NAD(P)H-dependent superoxide production was reduced by simvastatin in salt-induced hypertensive rats. Therefore, it is possible that the ability of simvastatin to prevent the detrimental effect of CRP is through the inhibition of NAD(P)H oxidase-induced superoxide production the blockade of RhoA/Rho-kinase activation, as suggested in our previous studies. Although a direct action of eNOS by simvastatin cannot be excluded, our present study does not support this view because intraluminal treatment with simvastatin alone for 60 minutes failed to enhance bradykinin-induced vasodilation.

In conclusion, we have demonstrated for the first time that CRP, at a concentration known to predict vascular disease, directly inhibits the endothelium-dependent NO-mediated dilation of isolated porcine retinal arterioles. The mechanism underlying the acute effect of CRP involves the activation of p38 kinase and the production of superoxide by vascular NAD(P)H oxidase. In addition, statins are beneficial by preserving endothelial function, possibly through the inactivation of the RhoA/Rho-kinase pathway and the reduction of oxidative stress. Because impaired endothelium-dependent NO-mediated dilation is a key feature of early vascular events, CRP is clearly not just an inflammatory marker but also a mediator for the development of vascular disorders in the retinal circulation. The results obtained from the present studies may help our understanding of the pathogenesis of retinal vascular disease associated with high levels of CRP.

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References


